Destabilization of Collagen in Hide and Leather by Anionic Surfactants. II. Calorimetry of the Reaction of Collagen with Sulfates

ABSTRACT: Leather, a textile based on collagen, usually requires the addition of sulfated oils that have been recently found to cause instability when heated in critical manufacturing processes. Here reactions between collagen and sodium dodecyl sulfate (SDS), sulfated castor oil, or a synthetic sulfated oil are studied calorimetrically. Sodium lauryl sulfate below its critical micelle concentration (cmc) displayed an immediate exotherm due to equilibrium binding of the reagents with stoichiometry $n = 12.6 \pm 0.2$, $K = (2.02 \pm 0.8) \times 10^7\ M^{-1}$, and enthalpy $\Delta H = 62 \pm 2$ Kcal/mol; and a delayed endotherm due to denaturation of collagen. The endotherms accompanying the reactions with sulfated oils with longer chains were smaller, with no apparent denaturation of collagen. The micellar nature of these surfactants was apparent from very large n for sulfated castor oil, 4082 ± 11 and a very small value of ΔH, 0.77 ± 0.01 cal/mol. The binding of sulfated castor oil at the polar bands of collagen crystallites, comprising extended molecules arranged side-by-side, was shown directly by electron microscopy. © 1998 John Wiley & Sons, Inc. J Polym Sci B: Polym Phys 36: 805–813, 1998
Keywords: collagen; surfactants; calorimetry; segment-long-spacing; anionic; titration; sodium lauryl sulfate; castor oil

INTRODUCTION

Collagen type I is a fibrous protein from which the oldest mass-produced nonwoven textiles were made. The basic product, parchment, is made by removing the noncollagenous constituents of hide or skin tissue and drying the rest. To make a practical product, leather, which unlike parchment is stable to heating, microbiological degradation, and moisture, and which has desirable textural properties, the collagen must be crosslinked. The usual cross-linking agent for the last century, nanoparticles of chromium oxide, requires the addition of special softening oils called "fatliquors," without which the dried product would still have the consistency of parchment. Most fatliquor is based on sulfated fish or castor oil.

The collagen I molecule itself has the form of a broken rod about 300 nm long, most of its length comprising three side-by-side helical chains of nearly equal length. The molecules are packed into regular fibrils 100 nm in diameter, which in turn form larger fibers with a wide distribution of diameters. Cross-links are placed among the molecular chains within fibrils, stabilizing them. Because of a few cross-links that exist near the nonhelical ends of the molecules in the native tissue, its fibers are insoluble but can be dissolved if the ends are removed enzymatically.

We have reported that sulfated oils, which are used almost universally for softening chrome-tanned leather, counteract the stabilizing effect of the chromium-oxide crosslinks, causing the leather to be unstable when heated in certain manufacturing processes (drying and stretching).[1] Because of the great importance of the interaction of collagen with these surfactants in this context, we here examine the nature of the reaction. The interactions of surfactant with other proteins vary; some hydrophobic proteins are actually thermally stabilized by binding to surfactants.[2] Because it is not globular, mechanistic models involving intermediate structures with local order[3] might not apply to collagen I. The thermal changes during the reaction are of particular interest because they invariably accompany configurational changes in proteins. In this work we follow the sequence of thermal exchanges when surfactants react with collagen at constant temperature.

MATERIALS AND METHODS

Collagen was the pepsin-digested bovine dermal product in 12 m*M* hydrochloric acid (Vitrogen-100™ Collagen Corp., Palo Alto, CA), (95–98% type I; rest, type III) or in citrate at pH = 3.6 (Pentapharm Ltd., Stamford, CT). The stock solutions were diluted and dialyzed versus 0.05*M* sodium citrate. The concentrations of the calorimeter solutions were determined by ultraviolet light absorption at 198 nm in hydrochloric acid.[4]

Sulfate/sulfonate ester surfactants from anionic fatliquors were extracted from Chemol-57™ synthetic oil (ChemTan Co., Exeter, NH) or from sulfated castor oil (generic from Reilly-Whiteman Co., Conshohocken, PA or Eureka 102™ from Atlas Chemical Co., Newark, NJ) by partition between water and ether.[5] The equivalent concentrations of surfactants were determined by two-phase titrations against the quaternary ammonium salt diisobutylphenoxyethoxyethyl dimethyl benzyl ammonium chloride (Hyamine 1622™, Rohm and Haas, Philadelphia, PA).[6]

Titrations were performed with an Omega titration calorimeter[7] (Microcal Inc., Northampton, MA). This instrument consists of a well-insulated vessel maintained at 30°C, containing 1.4 mL of the collagen solution. The long needle of a syringe (Hamilton Co., Reno, NE) with the surfactant solution is inserted into the vessel. The plunger of the syringe is driven by a computer-controlled stepping motor, measuring the precise times (10 min between additions) and volumes (5.5 μL). The needle of the syringe, flattened into a paddle at its end, is rotated at 400 rpm for stirring. Small changes of temperature that result from the intermittent reaction in the vessel are monitored with a thermopile and are recorded. Data analysis and curve-fitting were aided by the program Origin Version 2.9 (Microcal Inc., Northampton, MA).

Segment-long-spacing aggregates (SLS) were formed from soluble collagen by the procedure of Brodsky.[9] The stock collagen solution was diluted 100-fold into 0.1*M* acetic acid and was centrifuged at $10^5 \times$ g for 3 h. A dialysis bag with 20 mL of this solution was placed in a beaker with 0.1*M* acetic acid. Another dialysis bag containing 2 g adenosine triphosphate (ATP) in 10 mL of 0.1*M* acetic acid was also placed in the beaker. After standing overnight at 4°C, the collagen was harvested from the dialysis bag as an SLS precipitate in 4.5 m*M* ATP/acetic acid mother liquor.

For electron microscopy SLS in a drop of suspension was placed on a carbon-covered grid and allowed to settle onto it for 3 min. The supernatant solution was drawn away from the surface of the grid into filter paper and was replaced with a drop of surfactant extracted from sulfated castor oil (Eureka 102™) by the procedure described above, diluted 1 : 1 with the ATP-containing buffer used to make the SLS. After the excess surfactant solution had been drawn off, the SLS were washed 10 times with the ATP buffer and stained with either phosphotungstic acid (PTA) solution at pH = 4.0 or with osmium tetroxide vapor. Grids were also prepared without surfactant but treated with osmium tetroxide. The electron microscope was a Zeiss Model EM10B operated at 60 Kv.

RESULTS

Sodium Dodecyl Sulfate Binding

Figure 1 shows the heat flow when 19.4 m*M* SDS is injected sequentially five times into 10^{-2} m*M* collagen at 50°C. At this temperature, the collagen is completely denatured, so it is effectively gelatin. Each injection causes a sharp fall in temperature, followed by a 12 s relaxation, which is the response time of the instrument. The negative temperature pulses inform us that the reaction of

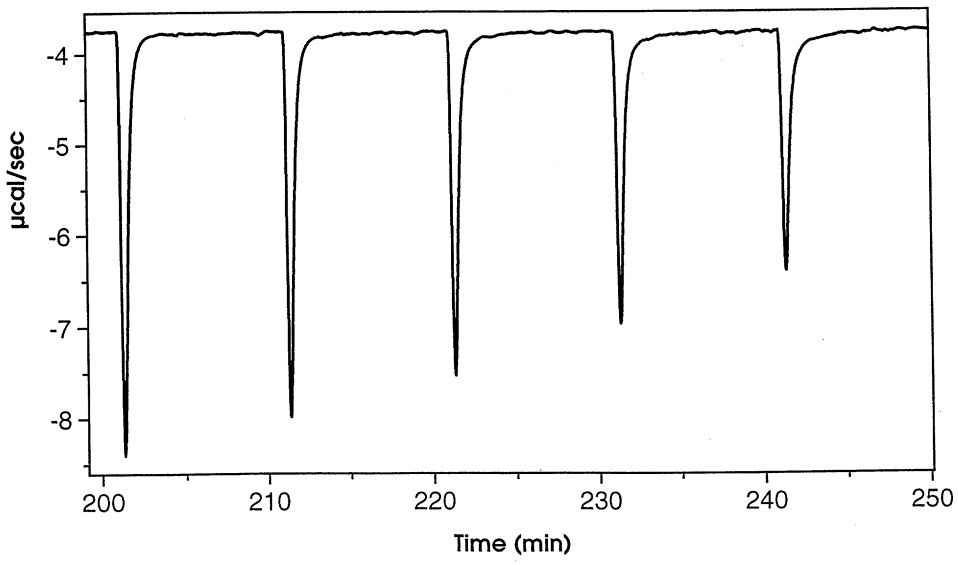

Figure 1. Thermal pulses from the reaction of SDS with collagen in solution at 50°C. Concentration of (a) collagen, 10^{-2} m*M;* (b) SDS in the syringe, 19.4 m*M;* pH = 5.0.

SDS with gelatin is endothermic; heat is absorbed from the surroundings, suggesting a reaction involving hydrophobic more than electrostatic bonds. This information will be useful in interpreting the response of native collagen to SDS.

The heat flow accompanying the titration of native collagen with SDS at pH = 2.7 and 30°C is shown in Figure 2. Over the whole titration, the concentration of SDS is kept below the critical micelle concentration (cmc) of 8.16 m*M.*[10] The course of the titration is divided into three regions, demarcated by lines drawn below the data curve.

The pulses in region 1 are simple like those of the titration of gelatin in Figure 1, but these are exothermic. Heat is evolved, as expected if the reaction is driven by the electrostatic forces of the cationic protein groups and the SDS anions. Further, unlike the gelatin-SDS system, the baseline is not flat, but rises slowly, showing a slow evolu-

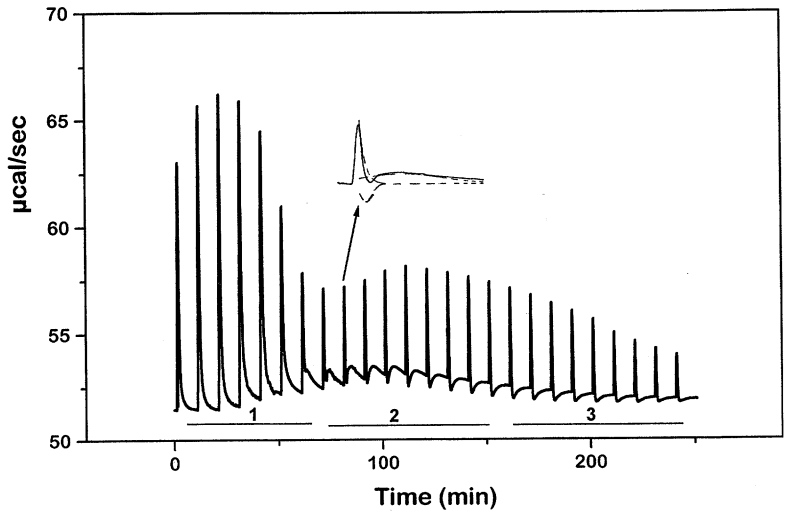

Figure 2. Thermal pulses during titration of collagen with SDS at pH = 2.7, 30°C. Concentration of collagen, 4.09×10^{-3} m*M;* of SDS in the syringe, 2.31 m*M.* Inset: expanded view of pulse No. 9, with its three components (see text) as broken lines.

tion of heat. We observed the gradual formation of a precipitate to accompany this slow evolution over the course of region 1. The end of this region, at the inflection of the baseline, can be taken as the end point of the titration of the ionic reaction, since it also coincides with a rapid decrease in the area and height of the exothermic pulses.

On region 2 of the titration, complex pulses display three components: a prompt exothermic reaction followed by an endothermic one, and then finally another exotherm. These are resolved and shown for pulse number 9 in the inset of Figure 2. We believe that the prompt exotherm still is due to the ionic reaction, accompanied and followed by endothermic denaturation of the reacted collagen. That the collagen becomes denatured under these conditions was previously shown by differential scanning calorimetry (DSC) of the precipitated product.[1]

The pulses on region 3 are similar to those of region 2 without the final exotherm, but follow each other with slowly decreasing areas and amplitudes.

To isolate the heat Q of the prompt binding reaction from each pulse, we fitted to each of them the function

$$Q = A \exp[(t - 46)/12] + (B/W_1)(2/\pi)^{1/2}\exp[(2/W_1)(t - 12)]^2 + (C/W_2)(2/\pi)^{1/2}\exp[(2/W_2(t - t_2)]^2 \quad (1)$$

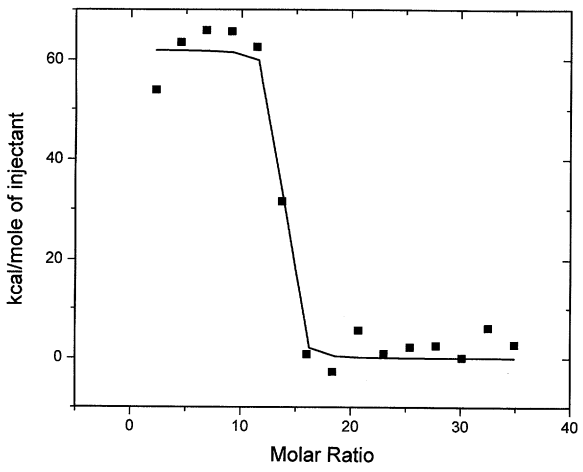

Figure 3. Thermal titration of collagen with SDS; data from Fig. 2. Points are experimental data; line is theoretical calculation for titration of one type of binding site on collagen.

Table I. Thermodynamic Parameters of Binding of Two Anionic Surfactants to Collagen from Figures 3 and 6

	SDS	Chemol-57™
n	12.6 ± 0.21	4082 ± 11
K (M^{-1})	(2.02 ± 0.8) × 10^7	1.3 × 10^7
ΔH (kcal/mol)	62 ± 2	0.77 ± 0.01

n = number of sites per molecule, K = association constant, ΔH = molar enthalpy of binding.

where

A = amplitude of the first exotherm, an empirical constant
W_1 = width of the endothermic peak, an empirical constant
B, C = areas of components 2 or 3 (empirical constants)
t_2 = maximum of the second exothermic peak, an empirical constant
W_2 = width of the second exothermic peak, an empirical constant

In the first term on the right, 46 and 12 are instrumental constants; in the second term, 12 is the consensus location of the observed negative peak.

The integrals of the second and third terms in eq. (1) were subtracted from the total area under the pulse to obtain the heat of the binding reaction for each injection of SDS.

The integrated heats of the initial components of the pulses are presented in Figure 3. This titration curve was fitted to a model reaction in which injected SDS binds to several sites of a single type on the collagen molecule. Its parameters are given in Table I.

The enthalpy change and association constant appear to be reasonable for this ionic reaction, but the stoichiometric molar ratio n is much smaller than the number of positive charges on the collagen molecule at low pH.

Binding of Surfactant to Collagen

Unlike SDS, the fatliquor surfactants were studied above their cmc's to achieve adequate sensitivity. Saturable binding again was observed, with evolution of heat (Fig. 4). Again, individual pulses were complex, with a small endotherm, ap-

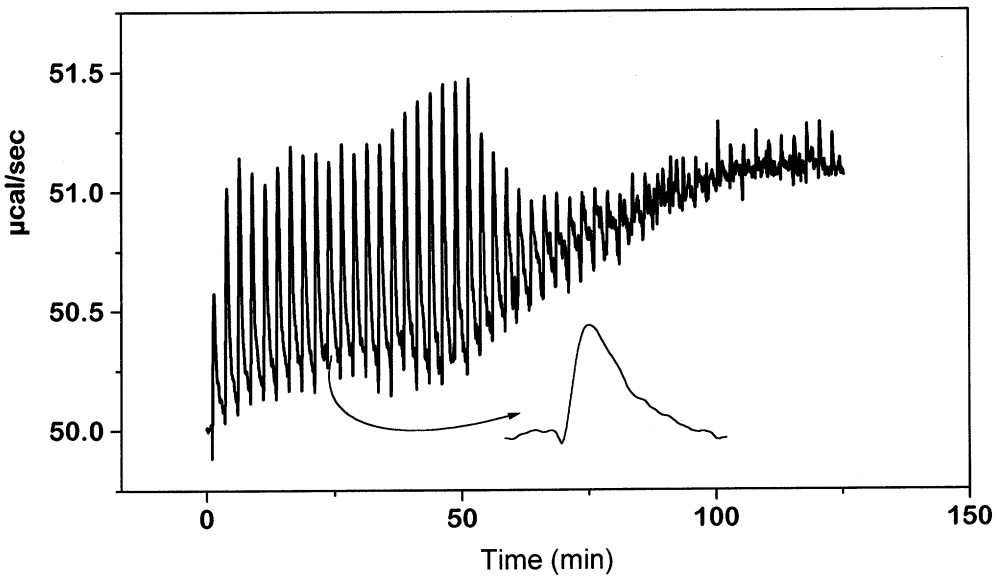

Figure 4. Thermal pulses during the titration of collagen with synthetic sulfated oil Chemol-57™ at pH = 5.0, 30°C. Concentration of collagen, 3.30×10^{-3} m*M;* of SDS in the syringe, 142 m*M*.

parently immediate, superposed on a much larger immediate exotherm with the instrumental decay rate, all overlapping a slowly decaying second exotherm (Fig. 4, inset). This behavior was also observed when the surfactant from sulfated castor oil (Reilly-Whiteman) was used (Fig. 5), but the prompt endotherm was larger.

Each pulse function Q was decomposed into two simultaneous exponential decays, which were fitted to the part of the pulse after the small initial endotherm:

$$Q = A_1\exp[(46 - t)/12] + A_2\exp[(46 - t)/t_1] + Y \quad (2)$$

where Y, A_1, A_2, and t_1, are empirical fitted constants, and the other constants are characteristic of the instrument. The first term describes the prompt binding that we seek; we integrated it above Y between 46 and 90 s to obtain the heat of binding for each pulse, excluding the second exotherm.

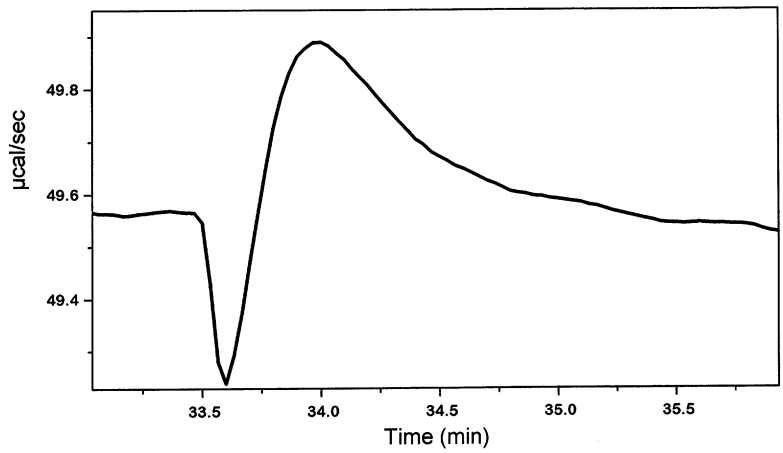

Figure 5. Thermal pulse from the reaction of collagen with sulfated castor oil at pH = 5.0, 30°C. Concentration of collagen, 6.60×10^{-3} m*M;* of SDS in the syringe, 4.02 m*M*.

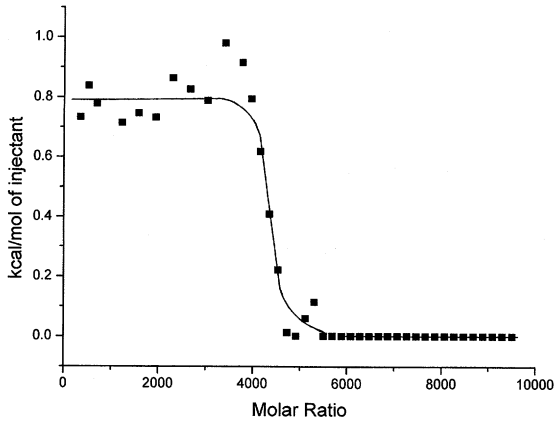

Figure 6. Thermal titration of collagen with Chemol-57™; data from Fig. 4. Points are experimental data; line is theoretical calculation for titration of one type of binding site on collagen.

The heats of reaction for the injections of Chemol-57™ are plotted in Figure 6. The parameters are shown in Table I.

Direct Observation of Surfactant Binding

To determine where on the collagen molecule the surfactant binds, we used a condensed form of solid collagen, segment-long-spacing crystallites (SLS),[10] in which the extended helical molecules are arranged side by side into flat rectangles (Fig. 7). In Figure 7a, these arrays have been stained with a heavy-metal anion, phosphotungstate (PTA), to make visible the regions where the ionic groups are clustered. Because the molecules are lined up side by side, these polar regions appear as many light bands across the molecular direction.

When we substituted sulfated castor oil from the surfactant for the heavy metal and then stained this with osmium tetroxide, only five wide bands were obvious, in a characteristic pattern (Fig. 7b). Osmium tetroxide alone does not develop the ionic bands, although it does bind to collagen (Fig. 7c).

It is not obvious from Figure 7a and 7b how the staining patterns from the surfactant anions and from the phosphotungstate anions are related. In Figure 8, however, we have plotted the microphotometer profile of the staining along the molecular length for representative SLS crystallites from each preparation. For data smoothing, the optical densities were averaged across the SLS (along the bands) at each point along the profile. Although the profile of the SLS with the PTA stain has a much more detailed structure, one can see five broad envelopes that match the five wide bands of the surfactant in SLS. The intensities of the bands, however, are not correlated between the two patterns. Since both preparations were treated with surfactant, the differences are not due to any attack of the surfactant on the registered ordering of the molecules.

DISCUSSION

Our data show that the reaction of surfactants with collagen, the main constituent of leather fibers, is complex and proceeds in stages (Figs. 2, 4, and 5). In the simple model that we chose for our data analysis, surfactant molecules bind immediately to sites on molecules of collagen in its native state, giving a positive (exothermic) heat pulse. Then an endothermic reaction follows in the vicinity of the binding site, which could be denaturation of collagen, leaving other sites intact. If the binding is weak, some surfactant remains free in the solution. With addition of more surfactant in the course of the titration, more surfactant binds, repeating the process with the unreacted binding sites, until no more unoccupied sites remain. Then the observed pulses diminish in area. From the titration curve, with the assumptions of this model, we can calculate n, the number of sites on the collagen molecule; K, the binding constant; and ΔH, the molar enthalpy of binding.

If, however, the binding of a small, nonsaturating, amount of surfactant causes extensive configurational changes in the collagen molecule, the unoccupied sites might change in number or in their affinities for surfactant. In the continuing titration, the additional surfactant would find binding sites that are different from those that reacted earlier: They might be stronger or weaker; they might be endothermic (if the collagen to which they belong has been denatured); or they might vanish. In these cases, none of the three thermodynamic parameters would be valid. The magnitude of ΔH would be too small; n, too small; and K, either too small or too large.

We observed small increases in the enthalpy of reaction during the first part of the titrations, before the end points (Figs. 2 and 6). Although we treated these maxima as experimental error, they are reproducible. This implies that the reac-

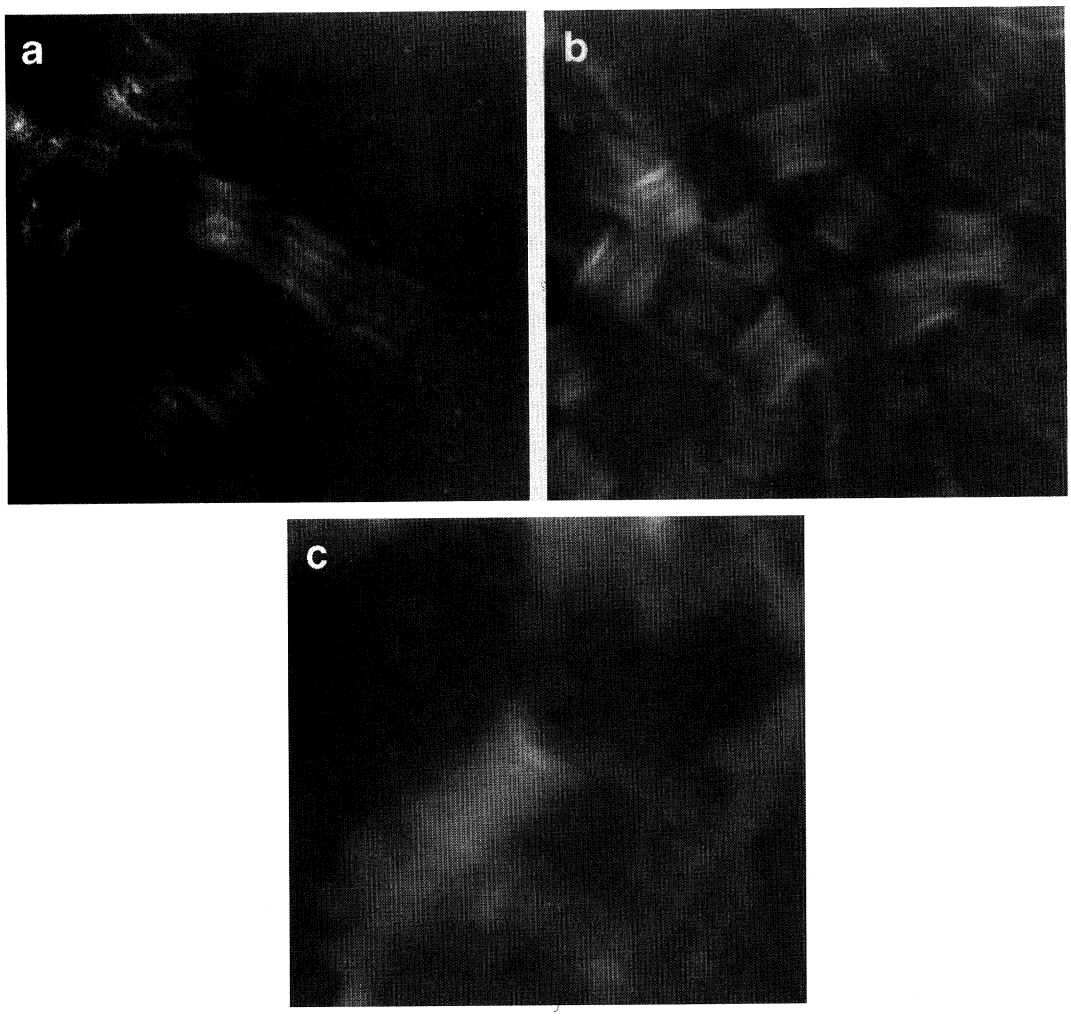

Figure 7. Transmission electron micrographs of SLS crystallites (a) treated with sulfated castor oil and osmium tetroxide; (b) treated with sulfated castor oil and stained with PTA; (c) stained with osmium tetroxide only.

tion is not producing more endothermic binding sites. Such sites would absorb heat, as shown in Figure 1, and cause the observed enthalpy to fall, not rise. The error in ΔH is therefore close to that given in Table I.

Because of the uncertainties in deconvolution, we did not correct ΔH for the heat of dilution. We determined that it amounted to only about 10% of the reported values, diminishing as the titration proceeded.

The meaning of the observed end point can be questioned. According to our model, it marks the exhaustion of binding sites in native collagen. On the contrary, it could mark the exhaustion of binding sites due to denaturation or precipitation of collagen. We observed, however, no endothermic component or precipitation in any of the pulses before the end point (Figs. 2 and 6). Only if unoccupied sites were affected thermally could their apparent number, n, and the binding constant, K, have been altered with no effect on ΔH. It would be difficult however to describe a change in the collagen molecule with such a property.

The first step in the reaction is binding of surfactant molecules to a collagen molecule. Our data show that this first step is exothermic, consistent with ionic interaction. Puzzling is the small apparent number of binding sites for SDS at pH = 2.7, only 12.6 per collagen molecule, although consistent with the result of Sato and Goto,[12] $\ll 100$ per collagen molecule. Apparently most of the ca. 150 cationic sites in the molecule are in excess. The 12 or 13 that did bind SDS in our experiment seem to be more reactive than others,

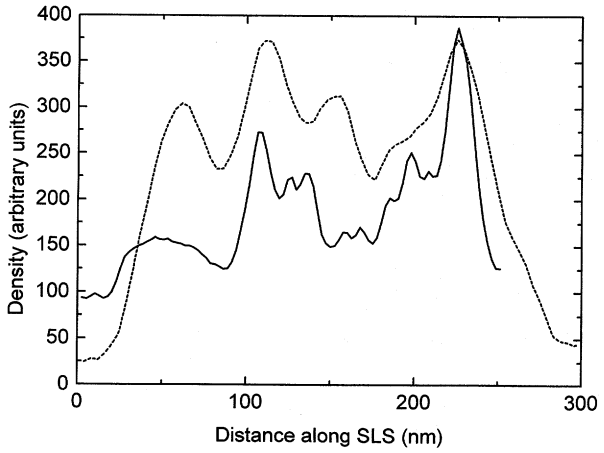

Figure 8. Densitometer scans perpendicular to the bands of SLS in Fig. 7. Solid line, SLS treated with sulfated castor oil and stained with PTA from Fig. 7a; dashed line, osmium (Fig. 7b).

which might cause the final series of exotherms that follow the end point of the titration (Fig. 2). The data at the tail of the titration curve, however, are too scattered to define a second titration step.

The second reaction step, with the slow endotherm appearing after the end point, could be due either to denaturation of the collagen or to binding to denatured collagen (as in Fig. 1). From the amount of collagen in the titration cell (1.72 mg) and the enthalpy of denaturation (60 J/g),[13] we expect the total heat change from denaturation alone to be -2.4×10^{-2} J. We observed -2.0×10^{-2} J, by adding together the integrals of all the observed endothermic components. We conclude that denaturation explains the endothermic component observed with SDS.

The behavior of the synthetic surfactant was very different from that of SDS, although the shape of the titration curve is similar. The number of molecular equivalents at the end point is much larger, 4082 ± 11. The result is again of the same order of magnitude that Sato and Goto found[12] for hexadecyl sulfate just below its cmc, 6000 binding to collagen at pH = 2. Such a large amount of surfactant binding to collagen and the small apparent enthalpy (0.77 Kcal/mol), uncorrected for the heat of dilution, indicates that it is in colloidal form, and that whole micelles bind. Only a few micellar molecules would interact directly with collagen. Since we have previously reported that the collagen in the complex is not denatured[1] by Chemol-57™, large micellar aggregates must be binding to intact collagen molecules.

It should be noted that the amount of this surfactant that binds at the end point corresponds to about three times the mass of the collagen, assuming an average molecular weight of about 200 for the surfactant. This end point is not even approached in practical leather manufacturing. The association constant, 1.3×10^7 M^{-1}, is close to that of SDS.

We did not correct the Chemol-57™ data for the endothermic component of the reaction. It would have lowered the net energy evolved that we measured, resulting in a lower apparent heat of reaction. Further, the molar heat of reaction was calculated on the basis of all the surfactant that we added as micelles, although only a small part of them could have found room to adsorb on a collagen molecule. (On the basis of the surface area of the collagen cylinder and the projection of the areas of 10 methylene groups needed for hydrophobic binding, we estimate a maximum of 600 surfactant molecules.) Since the molar enthalpy of binding, ΔH, was calculated on the basis of the total amount of surfactant that we added, the apparent value of 0.77 Kcal/mol could easily be too small by a factor of at least $4082/600 = 7$, but 100 would not be unreasonable. This is to say that a value of the enthalpy of 77 kcal/mol for the fatliquor would be consistent with our data and would resemble that for SDS.

The fatliquor works by migration of the surfactant from the oil droplets to the collagen, with coalescence of the oil, and is much more effective in this than SDS. One difference might lie in the number of hydrophobic bonds with the collagen. The "hydrophobic bond" is due to the entropy gained from the release of highly organized water from around the hydrophobic molecules; this displacement of water requires breaking some of its hydrogen bonds. Therefore, hydrophobic binding is endothermic,[14] opposing the ionic component of binding. The effectiveness of surfactant in fatliquor oil might actually be favored by a small value of ΔH due to partial compensation of ionic and hydrophobic energies of binding, suggesting that an effective surfactant should have a substantial hydrophobic component to its binding to collagen.

We observed directly (Fig. 7) the binding of a surfactant to collagen molecules for the first time. Quantitative comparison of Figure 7a with 7b through Figure 8 confirms that surfactant molecules bind to the polar regions of the collagen mol-

ecule. This hypothesis has been standing since the collagen-SDS studies of Gustavson.[15] Regions of the collagen molecule labeled with surfactant are large, appearing as 30-nm bands across the molecular axis. These regions are about five times as large as SDS micelles; their large size suggests that the particles of surfactant are also that much larger. This would be consistent with the large value of 4082 for n, the stoichiometry found by the calorimetric titration, and the facile coalescence of these fatliquor particles in the presence of collagen.

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REFERENCES AND NOTES

1. P. L. Kronick, *J. Am. Leather Chemists Assn.,* **91,** 246 (1996).
2. L. K. Creamer, *Biochemistry,* **34,** 7170 (1995).
3. O. B. Ptitsyn and G. V. Semisotnov, in *Conformations and Forces in Protein Folding,* B. T. Hall and K. A. Dill, Eds., American Association for the Advancement of Science, Washington, DC, 1992, p. 155.
4. G. C. Na, *Collagen Rel. Res.,* **8,** 315 (1988).
5. R. Hart, *Ind. Eng. Chem.,* **9,** 177 (1937).
6. G. F. Longman, *The Analysis of Detergents and Detergent Products,* John Wiley and Sons, New York, 1978.
7. T. Wiseman, S. Williston, J. F. Brandts, and L-N Lin, *Anal. Biochem.,* **179,** 131 (1989).
8. J. Gross and F. O. Schmitt, *J. Am. Chem. Soc.,* 3321 (1950).
9. B. Brodsky and E. F. Eikenberry, in *Methods in Enzymology,* Vol. 82, L. W. Cunningham and D. W. Fredericksen, Eds., 1982, pp. 127.
10. B. D. Flockhart, *J. Coll. Sci.,* **16,** 486 (1961).
11. J. Gross and R. R. Bruns, *Biochemistry,* **12,** 808 (1973).
12. K. Sato and S. Goto, *Das Leder,* **47,** 22 (1996).
13. P. L. Privalov and E. I. Tiktopulo, *Biopolymers,* **9,** 127 (1970).
14. C. Tanford, *The Hydrophobic Effect: Formation of Micelles and Biological Membranes,* John Wiley and Sons, New York, 1973, pp. 19–21.
15. K. H. Gustavson, *Acta Chim. Scand.,* **4,** 1171 (1950).